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12b. DISTRIBUTION CODE		13. ABSTRACT (Maximum 200 words) This study examined gene expression changes associated with exertional heat injury (EHI) in vivo and compared these changes to in vitro heat shock responses previously reported by our laboratory. Peripheral blood mononuclear cell (PBMC) RNA was obtained from four male Marine recruits (ages 17-19 yr) who presented with symptoms consistent with EHI, core temperatures ranging from 39.3 to 42.5°C, and elevations in serum enzymes such as creatine kinase. Controls were age- and gender-matched Marines from whom samples were obtained before and several days after an intense field-training exercise in the heat ("The Crucible"). Expression analysis was performed on Affymetrix arrays (containing 12,600 sequences) from pooled samples obtained at three times for EHI group (at presentation, 2-3 h after cooling, and 1-2 days later) and compared with control values (average signals from two chips representing pre- and post-Crucible samples). After post hoc filtering, the analysis identified 361 transcripts that had twofold or greater increases in expression at one or more of the time points assayed and 331 transcripts that had twofold or greater decreases in expression. The affected transcripts included sequences previously shown to be heat-shock responsive in PBMCs in vitro (including both heat shock proteins and non-heat shock proteins), a number of sequences whose changes in expression had not previously been noted as a result of in vitro heat shock in PBMCs (including several interferon-induced sequences), and several nonspecific stress response genes (including ubiquitin C and dual-specificity phosphatase-1). We conclude that EHI produces a broad stress response that is detectable in PBMCs and that heat stress per se can only account for some of the observed changes in transcript expression. The molecular evidence from these patients is thus consistent with the hypothesis that EHI can result from cumulative effects of multiple adverse interacting stimuli.		
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Exertional heat injury and gene expression changes: a DNA microarray analysis study

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Sonna, Larry A., C. Bruce Wenger, Scott Flinn, Holly K. Sheldon, Michael N. Sawka, and Craig M. Lilly. Exertional heat injury and gene expression changes: a DNA microarray analysis study. *J Appl Physiol* 96: 1943–1953, 2004. First published February 20, 2004; 10.1152/jappphysiol.00886.2003.—This study examined gene expression changes associated with exertional heat injury (EHI) in vivo and compared these changes to in vitro heat shock responses previously reported by our laboratory. Peripheral blood mononuclear cell (PBMC) RNA was obtained from four male Marine recruits (ages 17–19 yr) who presented with symptoms consistent with EHI, core temperatures ranging from 39.3 to 42.5°C, and elevations in serum enzymes such as creatine kinase. Controls were age- and gender-matched Marines from whom samples were obtained before and several days after an intense field-training exercise in the heat (“The Crucible”). Expression analysis was performed on Affymetrix arrays (containing ~12,600 sequences) from pooled samples obtained at three times for EHI group (at presentation, 2–3 h after cooling, and 1–2 days later) and compared with control values (average signals from two chips representing pre- and post-Crucible samples). After post hoc filtering, the analysis identified 361 transcripts that had twofold or greater increases in expression at one or more of the time points assayed and 331 transcripts that had twofold or greater decreases in expression. The affected transcripts included sequences previously shown to be heat-shock responsive in PBMCs in vitro (including both heat shock proteins and non-heat shock proteins), a number of sequences whose changes in expression had not previously been noted as a result of in vitro heat shock in PBMCs (including several interferon-induced sequences), and several nonspecific stress response genes (including ubiquitin C and dual-specificity phosphatase-1). We conclude that EHI produces a broad stress response that is detectable in PBMCs and that heat stress per se can only account for some of the observed changes in transcript expression. The molecular evidence from these patients is thus consistent with the hypothesis that EHI can result from cumulative effects of multiple adverse interacting stimuli.

heat stroke; exercise; peripheral blood mononuclear cells; genomics

EXERTIONAL HEAT ILLNESS encompasses a spectrum of clinical conditions, of which the most severe are exertional heat injury (EHI) and exertional heatstroke (EHS). EHI is defined as severe illness associated with high body temperatures in the setting of physical exertion, typically involving exhaustion or collapse, which is accompanied by evidence of tissue and organ damage (11). EHS is a more severe form of heat illness defined clinically by the presence of significant concurrent

central nervous system dysfunction (11). EHI and EHS account for many medical emergencies each year in athlete, worker, and military populations (25) and have recently received considerable attention because of a number of high-profile deaths among athletes.

The pathophysiological mechanisms that produce EHI and EHS are under active investigation (2). Experimental approaches have included investigations of animal mortality (10, 14), tissue damage (13, 17), and cellular factors [such as heat shock protein (HSP) expression] that affect tissue susceptibility to thermal injury (9, 16, 20). Animal models and observations in humans have identified mechanisms such as heat-induced translocation of lipopolysaccharide from the bowel lumen into the circulation (2, 12, 17, 22), reticuloendothelial system deactivation (4), and heat-induced changes in pro- and anti-inflammatory cytokines (2) as contributing to the pathogenesis of EHI and EHS. Additionally, human studies have examined the possibility that an inflammatory response induced by previous muscle injury leads to an accentuated hyperthermic response during exercise-induced heat loads (19). The picture that emerges from these mechanistic studies is that EHI and EHS share a common pathophysiological basis (3, 11) and represent systemic manifestations of derangements that occur at the level of cells and tissues, in which the immune system is highly involved (2). Identification of cellular pathways involved in EHI and EHS is a logical and important extension of this body of work. Until recently, this has been difficult because of limitations in our ability to simultaneously assay large numbers of mediator molecules in the context of thermal stress.

At the cellular level, it is generally accepted that thermal stress leads to increases in expression of HSPs and that the expression of these proteins correlates closely with the acquisition of thermotolerance (16, 18, 21). However, it is also increasingly apparent that the cellular response to heat shock involves more than HSPs (16, 28). For example, a recent DNA microarray study of rats exposed to whole body hyperthermia found substantial changes in liver expression of both HSPs and genes belonging to other functional classes (32). Similarly, isolated human peripheral blood mononuclear cells (PBMCs) subjected to in vitro heat shock demonstrated extensive changes in expression of both HSPs and genes not traditionally considered as HSPs (29).

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Knowledge of the molecular processes involved in EHI and EHS in humans has lagged behind our understanding of systemic processes, in part because it is difficult to obtain samples of human tissues thought to be of high potential interest, such as intestine, liver, skeletal muscle, and heart. Because leukocytes are easily obtained from human volunteers and show changes in HSP expression after heat shock *in vitro* (6, 7, 23, 24, 29) and after exercise *in vivo* (6–8, 23, 24), and because blood is exposed to all body compartments and thus all body temperatures (which are typically substantially elevated during EHI and EHS), PBMCs likely represent an informative cell type to identify molecular changes in EHI and EHS patients. Accordingly, we thought it likely that individuals suffering from EHI would demonstrate substantial changes in PBMC gene expression and that at least some of these changes would be similar to those found in human *in vitro* models of heat shock. This study, therefore, examined *in vivo* changes in PBMC gene expression associated with EHI and compared them to *in vitro* heat shock responses in PBMCs that were previously reported by our laboratory (29). We hypothesized that gene expression would be similar to heat shock but that many other genes associated with known systemic processes, particularly those related to inflammatory responses, would be expressed with EHI.

MATERIALS AND METHODS

Subjects and controls. The four EHI cases were male Marine recruits undergoing basic training at Parris Island, who presented to the field corpsman (medic) with evidence of heat illness and were evacuated to the Branch Medical Clinic Parris Island. The criteria for EHI included hyperthermia from physical exercise, with signs and symptoms such as collapse during exertion in the heat, malaise, disorientation, or ataxia, accompanied by laboratory evidence of organ dysfunction [such as elevated serum creatine kinase (CK), transaminases, or lactate dehydrogenase], a level of consciousness that returned to normal after cooling, and the absence of seizure activity. All subjects had active cooling started in the field (icing). On arrival to the clinic, they underwent further active cooling with ice sheets, typically followed by showering. In addition, all subjects received intravenous hydration. Blood samples were drawn on presentation, after cooling [mean \pm SD, 3.02 ± 0.24 (h:min) after presentation], and again on a follow-up visit 1–2 days later. Several blood samples were drawn on case 4 in the days after the initial scheduled follow-up. All cases were presented between July and September 2000. All four subjects had been in basic training at Parris Island long enough (range, 9–20 days from start of training to injury) to have become heat acclimatized.

Control subjects were three Marine recruits matched for age, gender, and ethnic group, who volunteered to donate blood samples in August 2000, several days before and several days after an intense field-training exercise ("The Crucible," the capstone event of US Marine Basic Training, which represents 54 h of vigorous field training with average energy expenditures of $>6,000$ kcal/day for men). Because this event was preceded by weeks of basic combat training in the summer heat, it is assumed that the controls were also heat acclimatized.

All Marine Corps recruits are medically screened before enlistment for an extensive battery of disqualifying conditions, including a variety of chronic liver diseases, infectious diseases such as chronic hepatitis, autoimmune conditions such as systemic lupus erythematosus that can produce renal and hepatic dysfunction, autoimmune conditions capable of producing rhabdomyolysis, alcohol dependence, and renal disease. Also disqualifying for enlistment are a known predisposition to heat illness, a prior history of malignant hyperther-

mia, recurrent episodes of heat injury requiring medical attention, and evidence of residual injury from a prior heat illness.

Blood collection. Blood was collected from all individuals in cell-preparation tubes (Fisher Scientific). Mononuclear cells were separated per the manufacturer's instructions, suspended in ~ 1 ml of RNALater (Ambion), and frozen at -80°C until RNA processing occurred.

RNA extraction. Samples were thawed, and the cells were pelleted by centrifugation. RNA was extracted by using the RNeasy Mini Kits (Qiagen). RNA yield was estimated by absorbance spectrophotometry, and the samples were stored at -80°C .

DNA microarray analysis. Due to limiting sample volumes and RNA yields, equal amounts of RNA from the samples obtained from the four cases were pooled. Similarly, equal amounts of RNA from the samples obtained from the three control subjects were pooled. DNA microarray analysis was performed by using Affymetrix U95Av2 gene chips as described previously (27, 29). Analyses using Affymetrix arrays (containing $\sim 12,600$ sequences) were performed from pooled samples obtained at each of the three time points for EHI group (1 chip each for samples obtained at presentation, after cooling, and at follow-up) and compared with control values (1 chip each for samples obtained before and after the Crucible) as described in Statistical analysis.

Expression measurement by RT-PCR. RT-PCR on the pooled samples was performed using standard techniques, as described previously (27, 29). The primers used for these reactions are listed (see Table 4).

Statistical analysis. The output signal from the Affymetrix U95Av2 array was preprocessed using MAS 5.0 software (Affymetrix). For each sequence on the array, the mean and standard deviation of the software-reported signals were calculated from the two pooled control chips. From these values, 98.33% population intervals were computed by adding or subtracting 2.39 standard deviations from the mean. Corresponding sequences from the heat-injury cases were considered to have a statistically significant deviation from the controls if their signals fell outside of these population intervals. Population intervals of 98.33% were chosen rather than 95% intervals to correct for the three multiple comparisons being performed; i.e., a P value of $0.05/3 = 0.0167$ was considered statistically significant. Other statistical analyses were performed as noted throughout the manuscript using SigmaStat 2.03 for Windows and taking a P value of ≤ 0.05 as statistically significant.

Where noted in this manuscript, post hoc filtering of significantly affected sequences was performed by two criteria. First, sequences had to show a twofold or greater change in expression. Second, sequences had to be detected by the chip-reading software as "present" or "marginal" in both control chips (for downregulated sequences) or in the chip(s) corresponding to the time point(s) of interest (for upregulated sequences). This post hoc filtering was performed to maximize the comparability of the results of this experiment with the analysis performed in our laboratory's previous *in vitro* experiment (29) involving five sets of normal human PBMCs subjected to heat shock in serum-free media. In this previous study, preprocessing was performed using MAS 4.0 software, and the post hoc filters used were a geometric mean change in expression of twofold or greater and a present or marginal call in five of five experiments in the controls (for downregulated sequences) or in the heat-shocked cells (for upregulated sequences).

RESULTS

EHI and control subjects. Table 1 provides the demographic characteristics of the EHI and control subjects. Tables 2 and 3 provide the clinical and laboratory characteristics of the EHI patients. Values outside the range of normal for the clinical laboratory are highlighted in bold font in Table 3.

Table 1. Demographic characteristics of cases and controls

	Ethnic Origin	Age, yr	Height, m	Weight, kg	BMI, kg/m ²	Time Between Presentation and Follow-up, days
Cases						
1	Caucasian	17	1.75	68.5	22.3	2
2	African-American	17	1.70	81.6	28.2	1
3	African-American	18	1.83	100.2	30.0	2
4	Caucasian	19	1.83	89.4	26.7	1
Mean \pm SE		18 \pm 0.5	1.78 \pm 0.03	84.9 \pm 6.7	26.8 \pm 1.6	1.5 \pm 0.3
Controls						
A	African-American	19	1.71	60.8	20.7	10
B	Caucasian	18	1.82	80.7	24.5	14
C	Caucasian	18	1.78	79.4	25.1	14
Mean \pm SE		18 \pm 0.3	1.77 \pm 0.03	73.6 \pm 6.4	23.4 \pm 1.4	13 \pm 1
P value	1.0*	0.40	0.88	0.29	0.20	<0.001

BMI, body mass index. *By Fisher's exact test. P values were derived using *t*-tests unless otherwise indicated.

All four EHI cases had at least one recorded body temperature in excess of 102.2°F (39°C) with their highest core temperatures ranging from 102.8°F (39.3°C) to 108.5°F (42.5°C) (as recorded either in the field or at the branch medical clinic). All subjects received initial cooling in the field with ice sheets and up to 14 min of additional treatment with ice sheets in the cool room of the acute care treatment area of the Branch Medical Clinic (Table 2).

Each of four EHI cases had prodromal symptoms in the days leading up to the acute event. The precipitating event involved running in three of the four cases and participation in an obstacle course in the fourth. No subject had neurological impairment more severe than mild confusion. All subjects had at least one blood sample showing elevation of unfractionated CK. With the exception of the fourth case, none of the cases had evidence of urinary heme pigment on the day of injury as judged by urine dipstick test. Case 4 had both a positive urinary dipstick test for heme pigment and a minor degree of microscopic hematuria on presentation (with 10–25 erythrocytes per high power field on urinalysis). Case 1 had evidence of mononucleosis (based on elevated serum Epstein Barr virus viral capsid IgM levels), case 3 had pharyngeal/tonsillar erythema and exudates on exam, and the chest radiograph from

case 4 was read by the treating practitioners as having an infiltrate in the left lower lobe that was felt to be suspicious for pneumonia. All cases displayed elevations of serum CK above normal, although only case 4 displayed an elevation of CK above 3,000, suspicious for clinically significant rhabdomyolysis. All four cases received intravenous hydration and showed a resulting decrease in serum creatinine. There were varying degrees of leukocytosis, with a relative shift in the distribution of cell types toward predominance of granulocytes (>80%) in the blood samples taken after cooling (Table 3). In summary, each of our case subjects met the definition of EHI, although a broad range of disease severity was represented.

Number of sequences affected by EHI. Of the ~12,600 sequences present on the U95Av2 chip, 3,605 and 4,478 sequences were expressed as "present" or "marginal" in the pooled controls (drawn before and after the field-training exercise, respectively). In the cases, 4,040 sequences were similarly expressed at presentation to the acute care treatment room, 4,056 were expressed after cooling, and 3,634 were expressed at the time of follow-up. These numbers are consistent with the number of sequences consistently expressed in previous *in vitro* experiments with human PBMCs (~3,700) that used an earlier version of the U95 array (29).

Table 2. Clinical characteristics of subjects experiencing exertional heat illness

Case	Prodromal Symptoms	Activity Precipitating EHI	Level of Consciousness (Initial Field Examination)	Maximum Recorded Temperature*		BMC Time on Ice Sheets, min†	Temperature at Time of Removal From Ice*		Hydration in BMC, liters	Other Pertinent Findings
				°F	°C		°F	°C		
1	URI \times 3 days, nausea	2-mile run	Normal	103.2	39.6	9	101.6	38.7	IV: 2–3	Positive serum Epstein-Barr virus viral capsid IgM
2	URI \times 2 days	Obstacle course	Slowed/confused	102.8‡	39.3	10	102.3	39.1	IV: 2–3 PO: 1	
3	URI \times 4–5 days	Training run	Normal	103.8	39.9	6	101.7	38.7	IV: 2 PO: 0.75	Pharyngeal/tonsillar erythema and exudates
4	Fever \times 2 days; fatigue for several days	3-mile run of the physical fitness test	Slowed/confused	108.5	42.5	14	102.5	39.2	IV: 2	Left-sided wheezing; left lower lobe pulmonary infiltrate suspicious for pneumonia on chest X-ray
Mean \pm SD				104.6 \pm 2.6	40.3 \pm 1.5	10 \pm 3	102.0 \pm 0.4	38.9 \pm 0.2		

BMC, Branch Medical Clinic; URI, upper respiratory illness; IV, intravenous; PO, oral; IgM, immunoglobulin M. *Oral or rectal; medical records did not always specify which. Maximum temperatures were recorded in the field except as noted. †In all cases, subjects were wetted down and treated with ice sheets in the field. ‡Recorded on arrival in the BMC; no field temperature was recorded.

Table 3. Clinical laboratory characteristics of subjects experiencing exertional heat illness

Case	Maximum Serum Creatine Kinase			Serum Creatinine, mg/dl*			Maximum Serum AST			Maximum Serum ALT			Maximum Serum LDH			Serum Glucose, mg/dl			WBC Counts and Differentials																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
	Value, IU/l	Occurred on	At follow-up	Value, IU/l	Occurred on	At follow-up	On presentation	After cooling	At follow-up	WBC count, 10 ³ /mm ³	Granulocytes, %	PBMCs, %	WBC count, 10 ³ /mm ³	Granulocytes, %	PBMCs, %	WBC count, 10 ³ /mm ³	Granulocytes, %	PBMCs, %	At presentation		After cooling		At follow-up																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		

AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; WBC, white blood cell; PBMCs, peripheral blood mononuclear cells; N/A, not available. Ranges of normal for this reference laboratory are: creatinine kinase, 22–269 IU/l; creatinine, 0.5–1.5 mg/dl; serum ALT, 10–60 IU/l; serum LDH, 100–242 IU/l; serum glucose, 70–110 mg/dl; WBC count, 4.0–11.0 × 10³/mm³; % granulocytes, 42.2–75.2%. Values outside the range of normal for the clinical laboratory used are in bold. *Changes over time were statistically significant by one-way, repeated-measures ANOVA ($P=0.038$).

Although the absolute number of sequences expressed during EHI did not differ significantly from those expressed at baseline, the number of sequences that showed significantly different expression between cases and controls was large. As shown in Table 5, ~1,800 sequences showed a statistically significant increase in expression at each of the time points examined in the subjects with EHI, and ~2,000 sequences showed a significant decrease in expression. However, most of these changes in expression were relatively small, with only ~800 sequences showing increases of twofold or greater at each of the time points and a similar number showing decreases (Table 5). Even fewer sequences met our presence/absence post hoc filter criteria; in total, we estimate that 361 sequences were upregulated at one or more time points and that 331 sequences were downregulated.

The sequences that were significantly affected by EHI changed as a function of time. As illustrated by the Venn diagrams in Fig. 1 (center of each diagram), only a minority of sequences were significantly upregulated (or downregulated) at all three time points. A substantial number sequences showed a time-dependent response; for example, HSPs were typically most highly upregulated on the day of injury and had diminished or returned to control levels by the time of follow-up (Table 6).

We classified all sequences that were significantly affected and met our post hoc filter criteria into broad functional classes. For purposes of classification and illustration, each sequence was assigned to only one primary functional class. Of the 361 upregulated sequences, about three-fourths fell into one of the following classes: immune function, 85 sequences (24%); cell growth, proliferation, differentiation, and apoptosis, 37 sequences (10%); metabolism and redox control (including heme oxygenase-1), 33 sequences (9%); transcription, 29 sequences (8%); unknown, 29 sequences (8%); protein degradation (including ubiquitins, proteases, and antiproteases), 23 sequences (6%); HSPs, chaperonins, and cochaperonins, 21 sequences (6%); and signal transduction, 22 sequences (6%). Of the 331 downregulated sequences, about two-thirds fell into one of the following classes: unknown, 58 sequences (18%); immune function, 42 sequences (13%); cell growth, proliferation, differentiation, and apoptosis, 44 sequences (13%); transcription, 41 sequences (12%); signal transduction, 32 sequences (10%); and metabolism and redox control, 18 sequences (5%).

Heat shock response. EHI produced a strong, time-dependent heat shock response in PBMCs. As shown in Table 6, a significant change in expression was found in at least one representative member of most major families of known human HSP. As might be expected from a heat-induced process, the number of significantly upregulated HSP sequences was greatest at presentation and diminished over time. At the time of follow-up, only eight HSP sequences were still significantly upregulated, and, of these, only two showed a change in expression that was twofold or more greater than controls. The finding of a widespread increase in HSP expression is highly congruent with our laboratory's previous microarray study of the effect of in vitro heat shock on gene expression in PBMCs (29), and as noted in Table 6, almost all of the HSP sequences upregulated by EHI in vivo had previously been found to be upregulated in our in vitro heat shock experiments.

Table 4. PCR Primers used

Gene	GenBank No.	PCR Product Size, bp	Forward Primer	Reverse Primer
PSR	AJ950382	345	5'-GCATTGGTAGCAGAGGAAA-3'	5'-GCTCCAGTTCGTGAGACTCC-3'
IFI-60	AF026939	427	5'-AGGAAGGGTGGACACAACG-3'	5'-TAGCCATTGTTGGTGTGGA-3'
IFI-27	X67325	305	5'-GCCTCTGCTCTCACCTCATC-3'	5'-GAGAGTCCAGTTGCTCCGAG-3'
Unknown transcript	AB000115	321	5'-TCAGATTGGAACTGGACCC-3'	5'-TCAGGTTTGGCCTTTGAAC-3'
HSP70B'	X51757	579	5'-TGCCCGCCTATTTCATG-3'	5'-CCCCACCAGGACGAC-3'
Cyclophilin A	BC005982	406	5'-AGGTCCCAAGACAGCAGAA-3'	5'-TGTCACAGTCAGCAATCGT-3'

PSR, phosphatidylserine receptor; IFI, interferon inducible; HSP, heat shock protein.

Control sequences. Although EHI produced extensive changes in gene expression, these changes in expression were not universal. Table 7 shows the effect of EHI on a number of control sequences, including β -actin, GAPDH, cyclophilin A, and the five ribosomal protein sequences most highly expressed in the control subjects. Although some of these sequences showed changes in expression that were significantly different from the controls, none showed an absolute change of twofold or greater, the cutoff used in our post hoc filtering process.

Effect of EHI on non-HSPs. The non-HSPs most strongly (≥ 5.0 -fold after rounding, at one or more time points) affected by EHI are listed in Tables 8 and 9. Importantly, of the 36 upregulated sequences listed in Table 8, at least one-fourth are known to be interferon inducible. Interestingly, in comparing these results to previous in vitro data (29), only two of these non-HSPs had previously been found to be significantly upregulated after an in vitro heat shock delivered to normal PBMCs ($43^\circ\text{C} \times 20$ min followed by 2 h 40 min of recovery at 37°C): the phosphatidylserine receptor (upregulated 3.1-fold in vitro) and pyruvate carboxylase (upregulated 1.8-fold in vitro but did not meet our post hoc filter criteria in the previous study). Furthermore, seven of these upregulated genes were actually downregulated in our previous in vitro experiment: granzyme B (down 0.49-fold in vitro), adrenomedullin (down 0.47-fold in vitro), complement component 3a receptor 1 (down 0.40-fold in vitro), myxovirus resistance A (down 0.23-

fold in vitro), endothelial cell growth factor 1 (down 0.38-fold in vitro), interferon inducible protein 60 (down 0.12-fold in vitro), and interferon-induced protein with tetratricopeptide 2 (down 0.22-fold in vitro but did not meet the previous study's post hoc filter criteria). Among the differences between the two experiments, it is noteworthy that the in vitro experiment was performed in serum-free media.

A sequence-encoding interferon- γ (GenBank no. X13274) was significantly upregulated by EHI, with changes of 3.7-fold at presentation, 3.0-fold after cooling, and 7.6-fold at follow-up. However, this sequence showed a low level of expression and did not meet our post hoc presence/absence filter criteria. In our laboratory's previous in vitro experiment (29), this sequence was upregulated 4.3-fold, but the variance from experiment to experiment was high enough to render the change not statistically significant (increased in 4 experiments, decreased in 1 experiment; 95% confidence interval, 0.66 to 28). Thus, both in vitro and in vivo, our microarray evidence is equivocal but favors the possibility that heat stress leads to increased expression of interferon- γ mRNA.

Two interferon- α sequences on the Affymetrix array (GenBank V00541 and X02956, both encoding interferon- α_5) showed statistically significant increases of twofold or greater at presentation (2.4-fold and 2.1-fold, respectively) but not after cooling or at recovery. However, neither of these sequences met our post hoc criteria for inclusion, and neither had been upregulated in our laboratory's previous in vitro study (29).

Among the 35 most strongly downregulated sequences, only 5 were previously found to be downregulated as a result of in vitro PBMC heat shock (Table 9). It is noteworthy that the time point at which maximum downregulation occurred was most commonly at follow-up (1–2 days after initial presentation), which is substantially later than the time point examined in the in vitro experiment (2 h 40 min after heat shock).

Confirmatory RT-PCR. RT-PCR was performed on a select number of genes identified by the microarray as being strongly upregulated by heat shock (Fig. 2). The source material for this PCR came from control subject B (lanes 1 and 2) and case 2 (lanes 3–5). As illustrated, the PCR confirmed the presence of an increased expression of HSP70B', phosphatidylserine receptor, interferon-induced proteins 27 and 60, and AB000115 (an open reading frame on chromosome 1) in the EHI case. By contrast, there was no clear effect on cyclophilin A, as predicted by the microarray results.

Comparison to previous in vitro heat shock in PBMCs. Previous work in our laboratory (27) has suggested that human cells display a relatively small nonspecific response to environmental stress in addition to stress- and cell type-specific

Table 5. Number of sequences affected by exertional heat illness

Time Point	Direction of Change	Number of Sequences Showing:		
		Statistically significant difference in expression*	... and a signal difference of ≥ 2 -fold*	... and also met our post hoc presence/absence filter criteria
At presentation	Any	3615		
	Up	1786	767	144
After cooling	Down	1829	717	115
	Any	3900		
At follow-up	Up	1742	760	237
	Down	2158	966	195
At any time point	Any	3923		
	Up	1895	856	179
	Down	2028	917	176
	Any†	5558		
	Up	3147	1555	361
	Down	3179	1675	331

*Relative to matched controls. †Total does not equal the sum of "up" plus "down," because some sequences showed a biphasic response (e.g., up at 1 or more time points, down at others).

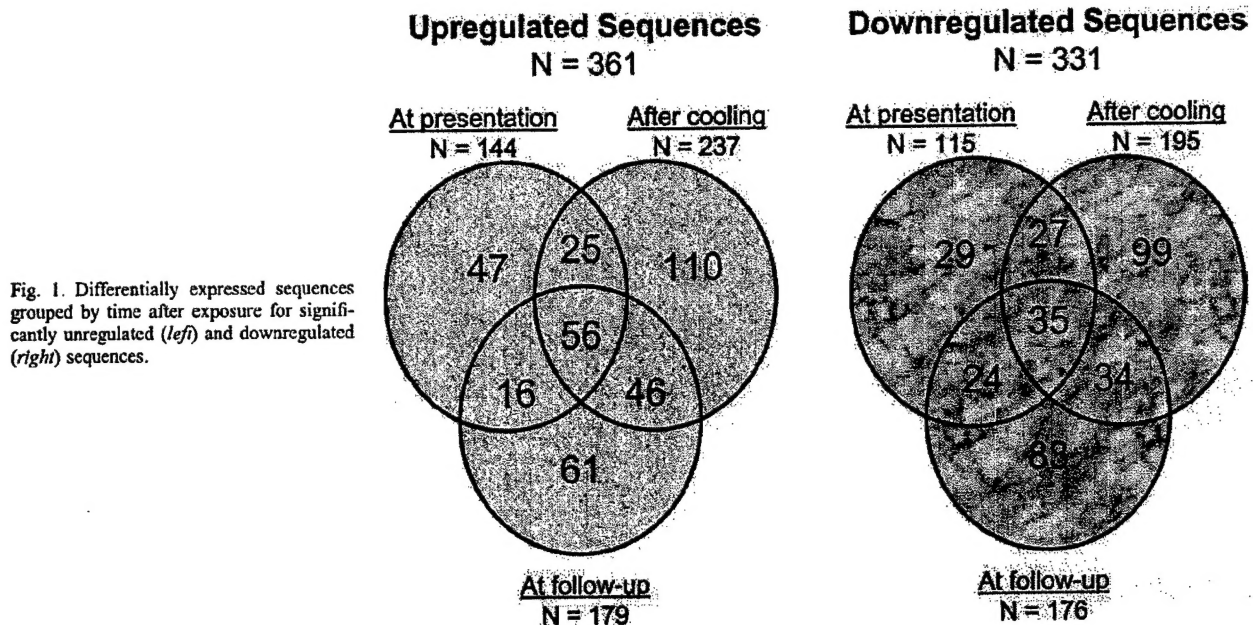


Fig. 1. Differentially expressed sequences grouped by time after exposure for significantly upregulated (left) and downregulated (right) sequences.

responses. From the data in Tables 6, 8, and 9, it became apparent that the PBMCs of the subjects with EHI had evidence of not only a conventional heat shock response (defined as increased expression of HSPs and other genes previously found to be upregulated by heat shock in vitro) but also of a response that differed from the previously observed in vitro effects of heat shock on PBMCs. To better estimate the extent to which our in vivo responses could be accounted for by thermal stress alone, we compared the list of 361 upregulated and 331 downregulated genes to the list of all genes significantly affected by heat shock in our in vitro PBMC experiment (29). Similarly, to estimate the magnitude of the stress non-specific response (previously estimated at ~10–15% of all sequences affected), we compared the changes in expression noted here to those found in a database of cells exposed to a nonthermal stress (HepG2 cells exposed to hypoxia in vitro for 24 h) (27). To reduce the biasing effect of post hoc filtering, we included all sequences from the in vitro experiments that showed a statistically significant change in expression, independent of the magnitude of change or the presence/absence calls.

Of the 144 sequences upregulated at presentation, 35 (24%) had previously been found to be significantly upregulated by in vitro heat shock. By contrast, only 13 (9%) were similarly affected by hypoxic exposure in HepG2 cells ($P < 0.001$ by χ^2 analysis). Of the 237 sequences upregulated after cooling, 41 (17%) were similarly affected by in vitro heat shock, which was comparable to the number of sequences affected by hypoxia [34 (14%), $P = 0.45$]. When we compared the 179 sequences that were upregulated at recovery, we found that only 17 (9%) were similarly affected by in vitro heat shock and 15 (8%) by hypoxia ($P = 0.85$). Although these numerical findings would suggest an overlap between the three studies of ~10–15%, when the actual sequences affected were compared, only eight sequences representing seven unique genes were significantly upregulated in all three studies at one or more

time points. These included the well-established stress genes ubiquitin C, dual-specificity phosphatase-1, and colligin 2 (HSP47), as well as the phosphatidylserine receptor, calcium-binding protein S100A2, pyruvate carboxylase, and the collagen synthesis enzyme lysine hydroxylase.

Among the downregulated sequences, 11–18% have previously been found to be significantly downregulated by in vitro heat shock in PBMCs or by hypoxic exposure in HepG2 cells. Neither of the two in vitro experiments showed a significantly greater degree of overlap than the other with the effects of EHI.

DISCUSSION

Our most important finding is that the PBMC gene expression response to EHI is far more extensive than previously recognized. This response appears to include a time-dependent series of changes that has at least three components: 1) a heat shock response that involves elements such as HSPs, as described in PBMCs and other cell lines in vitro; 2) a response that includes a number of genes not previously found to be upregulated by heat shock in our in vitro experiments in PBMCs and that includes a substantial number of interferon-inducible genes; and 3) a small nonspecific stress response that is shared by other cell lines and stressors.

Although limited by several technical considerations (discussed below), the findings reported here are biologically plausible within the context of what is known about thermal stress in vitro and in vivo. For example, a time-dependent heat shock response was observed, a phenomenon that has been noted in vivo in contexts such as intense exercise (7, 8). It is also noteworthy that a sequence corresponding to c-myc was among the most highly downregulated sequences at presentation, because in vitro experiments have found that decreased expression of this gene is essential to the process of cellular recovery from severe thermal stress (31). Additionally, the congruence between the findings of the present study and our

Table 6. HSPs affected by exertional heat illness

Family	Common Name(s)	GenBank or TIGR No.	Fold-Change in Expression (Subjects/Controls)			Effect of In Vitro Heat Shock
			At presentation	After cooling	At follow-up	
HSP10	HSPE1; heat shock 10-kDa protein 1; chaperonin 10; HSP10	A1912041	2.3	3.8	0.40	Up
HSP27	HSPB1; Heat shock 27-kDa protein 1; HSP28; HSP27-1	Z23090	9.6	32	1.1 (NS)	Up
HSP32	Heme oxygenase-1; HSP32	Z82244	3.8	3.3	1.4	Up
HSP40	DNAJA1; DnaJ (HSP40) homolog, subfamily A, member 1; HSPF4	L08069	3.5	1.4 (NS)	1.2 (NS)	Up
	DNAJA1; DnaJ (HSP40) homolog, subfamily A, member 1; HSPF4	L08069	2.1	1.3 (NS)	0.93 (NS)	Up
	DNAJB1; DnaJ (HSP40) homolog, subfamily B, member 1; Hsp40	D85429	8.4	1.5 (NS)	0.71 (NS)	Up
	DNAJB6; DnaJ (HSP40) homolog, subfamily B, member 6; MRJ	A1540318	2.5	1.9	1.7	Up*
HSP47	HSP47; colligin 2; SERPINH2	D83174	6.6	7.4	0.66 (NS)	Up
	HSP47; colligin 2; SERPINH2	D83174	1.5	1.6	0.40	Up
	HSP47; colligin 2; SERPINH2	D83174	1.5	1.6	0.40	Up
HSP56	FKBP4; FK506 binding protein 4, 59-kDa; immunophilin; FKBP52; HSP56	M88279	5.2	6.7	1.6 (NS)	Up
HSP60	HSPD1; Heat shock 60-kDa protein 1 (chaperonin); HSP60-1; mitochondrial matrix protein P1	M22382	3.3	5.4	0.85 (NS)	Up
HSP70	HSPA1A and HSPA1B; Heat shock 70-kDa protein 1A; HSP70-1; heat shock 70-kDa protein 1B; HSP70-2	W28645	2.9	2.0	1.3 (NS)	Up
	HSPA1A; heat shock 70-kDa protein 1A; HSP70-1	M11717	31	22	1.2	Up
	HSPA1B; Heat shock 70-kDa protein 1B; HSP70-2	M59830	32	19	1.1 (NS)	Up
	HSPA6; heat shock 70-kDa protein 6; HSP70-B'	X51757	25	3.6	3.8	Up
	HSPA6; heat shock 70-kDa protein 6; HSP70-B'	X51757	20	3.8	2.3	Up
	HSPA8; heat shock 70-kDa protein 8; HSC70	HT2995	2.9	1.6 (NS)	0.85 (NS)	Up
	HSPA8; heat shock 70-kDa protein 8; HSC70	HT2995	2.0	1.1 (NS)	0.80	Up
	HSPA9B; heat shock 70-kDa protein 9B; mortalin 2	L15189	0.81	0.86 (NS)	0.47	Up*
	HSPA9B; heat shock 70-kDa protein 9B; mortalin 2	L15189	0.81	0.86 (NS)	0.47	Up*
HSP75	TRAP1; heat shock protein 75; HSP75; tumor necrosis factor type 1 receptor associated protein	U12595	0.54	0.39	0.89 (NS)	NS
HSP90	HSPCA; heat shock 90-kDa protein 1, alpha; HSP90-1-alpha	X15183	5.8	6.8	1.1 (NS)	Up
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta; HSP90B	J04988	3.1	3.3	0.76	Up
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta; HSP90B	M16660	2.6	2.8	0.68 (NS)	Up
Other	FKBP5; FK506 binding protein 5; HSP90-binding immunophilin	U42031	0.68 (NS)	2.1	1.2 (NS)	Up*
	STIP-1; HSP70/HSP90 organizing protein; stress-induced phosphoprotein 1	M86752	2.2	2.2	1.2 (NS)	Up
Ubiquitins	UBB; ubiquitin B	U49869	2.1	1.4 (NS)	1.8	Up
	UBC; ubiquitin C	M26880	2.8	2.2	1.7	Up*
	UBC; ubiquitin C	AB009010	2.3	1.8	1.6	Up*
	UBC; ubiquitin C	AB009010	2.3	1.8	1.6	Up*
Upregulated			25	20	8	27
Downregulated			2	1	5	0
Not significantly affected			1	7	15	1

Changes in gene expression were statistically significant unless otherwise noted. The sequences reported met our post hoc filter criteria at one or more time points. Some of the genes in this table can be classified into more than one major functional category. NS, not significant. *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria (listed in MATERIALS AND METHODS).

Table 7. Control sequences

Common Name(s)	GenBank No.	Fold-Change (Relative to Control)		
		At presentation	After cooling	At follow-up
Cyclophilin A	X52851	1.1	0.94	1.2
β -actin, 5' sequence	X00351	0.98	1.1	1.6*
β -actin, middle sequence	X00351	1.2*	1.2*	1.7*
β -actin, 3' sequence	X00351	1.2*	1.3*	1.5*
GAPDH, 5' sequence	M33197	1.0	1.3	1.4
GAPDH, middle sequence	M33197	1.0	1.5*	1.4*
GAPDH, 3' sequence	M33197	1.1	1.5*	1.5*
Ribosomal protein L41	Z12962	0.94	1.0	1.2
Ribosomal protein S29	A1541542	1.0	1.0	1.1
Large ribosomal protein P1	M17886	0.71†	0.84	1.1
Ribosomal protein S12	AA977163	0.84†	0.86	0.90
Ribosomal protein L38	Z26876	0.76	0.81	1.0

The list includes β -actin, GAPDH, cyclophilin A, and the 5 ribosomal protein mRNA sequences most highly expressed in the controls. * $P \leq 0.010$. † $0.010 < P < 0.0167$.

previous in vitro study in PBMCs was greatest for the samples obtained on the day of presentation (17–24%), as would be expected given the time point used in the in vitro study (2 h 40 min after heat shock). Importantly, the observed differences between the in vivo and in vitro responses suggest that, as might be anticipated from a multifactorial process, EHI also involves pathways other than those affected by in vitro heat shock in serum-free media. The molecular data reported here likely reflect not only the direct cellular effects of thermal stress on PBMCs but also the responses of PBMCs to the humoral or other signals detectable by cells traveling through tissues reacting to thermal stress.

It is noteworthy that many of the sequences most highly increased by EHI in this report are interferon-inducible genes (Table 8). Interferons- α and - γ have been variably reported to be elevated in exertional heat illness (reviewed in Ref. 2), and these immune modulators are known to be capable of producing flu-like syndromes, at least when administered at pharmacological doses (1, 15). As is commonly observed in EHI (5, 26), all of our cases reported feeling ill in the days leading up to the acute events, and at least one of the case subjects had

Table 8. *Non-HSP genes most strongly upregulated by EHI*

Gene Type	Common Name(s)	GenBank No.	Fold-Change (Subjects/Controls)			Effect of In Vitro Heat Shock
			At presentation	After cooling	At follow-up	
Maximally upregulated at presentation						
Immune function	GZMB; granzyme B; granzyme 2	M17016	5.6	1.8 (NS)	2.3	Down
Phagocytosis	PSR; Phosphatidylserine receptor	AI950382	5.2	2.9	1.6 (NS)	Up
Membrane transport	SLC15A1; Solute carrier family 15 (oligopeptide transporter), member 1; peptide transporter HPEPT1; PEPT1	AB001328	5.8	1.0 (NS)	4.5	NS
Maximally upregulated after cooling						
Cell adhesion	Cadherin	L43366	7.0	7.2	6.3	NS
	Ninjurin 1; nerve injury-induced protein-1; NINJ-1	U91512	1.2 (NS)	7.2	3.3	NS
Histone	H2AFO; H2A histone family, member O	AI885852	11	22	18	NS
Hormone	Adrenomedullin precursor	D14874	3.2	5.3	2.4	Down
Immune function/ immunoglobulin receptor	FCGR1A; Fc fragment of IgG, high affinity Ia, receptor for (CD64); CD64; Fc-gamma receptor 1A1	M63835	3.4	11	8.6	NS
Immune function/ complement receptor	C3aR1, complement component 3a receptor 1	U62027	3.3	8.6	3.0	Down
Immune function/ interferon inducible	IFI27; p27	X67325	2.8	40	21	NS
	G1P2; (clone IFI-15K)UCRP; IFI15; ISG15; interferon-stimulated protein, 15 kDa	M13755	5.0	6.7	5.1	NS
	Mx1; myxovirus-resistance 1; interferon-inducible protein p78; MxA	M33882	4.3	5.6	5.3	Down
	IFIT1; interferon-induced protein with tetratricopeptide repeats 1; interferon-induced protein 56	M24594	4.0	5.6	5.3	NS
		M24594	3.3	5.0	4.1	NS
Membrane function	Scramblase; phospholipid scramblase 1	AB006746	4.5	5.3	4.3	NS
Membrane transport	NEDD4L; neural precursor cell expressed, developmentally down-regulated 4-like	AB007899	2.8	5.0	1.3 (NS)	NS
Protein assembly	SCO2; SCO cytochrome oxidase deficient homolog 2 (yeast)	AL021683	4.5	5.1	5.1	NS
RNA stability and degradation	OASL; 2'-5'-oligoadenylate synthetase-like; thyroid hormone receptor interactor 14; TRIP14; p59OASL	AJ225089	6.1	9.5	8.5	NS
		L40387	2.4 (NS)	5.0	3.8	NS
Unknown	AB000115; C1orf29; chromosome 1 open reading frame 29	AB000115	6.2	7.8	5.7	NS
	AIP1; atrophin-1 interacting protein 1	AB014605	2.8	5.3	0.49 (NS)	NS
Maximally upregulated at follow-up						
Cell growth, proliferation, and differentiation	PPP2R1B; protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	M65254	2.3	8.2	12	NS
Growth factor	ECGF1; endothelial cell growth factor 1 (platelet-derived)	M63193	2.7	3.5	5.4	Down
Immune function/ interferon inducible	cig5; viperin	AF026941	9.9	20	20	NS
	IFI 60; IFIT 4; interferon-induced protein with tetratricopeptide repeats 4	AF026939	9.1	16	19	Down
	IFIT2; interferon-induced protein with tetratricopeptide repeats; 2G10P2; IFI54; cig42; IFI-54; GARG-39; ISG-54K	M14660	6.8	8.5	9.2	Down*
	CXCL10; chemokine (C-X-C motif) ligand 10; interferon-inducible cytokine IP-10	X02530	1.4 (NS)	1.2 (NS)	8.4	NS
	IFITM3; Interferon induced transmembrane protein 3 (1-8U)	X57352	4.2	5.5	6.9	NS
Immune function/ complement cascade	Complement component C1 inhibitor	X54486	4.3	5.4	7.6	NS
Metabolism	PC; pyruvate carboxylase	S72370	1.2 (NS)	2.3	7.7	Up*
Oxygen transport	HBE1; Hemoglobin, epsilon 1	AJ349593	3.2	3.9	5.1	NS
Signal transduction	Tyrosine phosphatase DKFZP566K0524	AL050040	5.7	3.4	7.3	NS
	ADORA2B; adenosine A2b receptor	X68487	3.2	3.6	6.0	NS
Translation termination/ interferon inducible	WARS; tryptophanyl tRNA synthetase	X59892	2.1	2.2	5.3	NS
Unknown	ZAP3; ZAP3 protein	L40396	5.3	3.9	8.0	NS
	SPUF; Secreted protein of unknown function	AA883101	4.4	4.1	5.4	NS

Included are all genes that met our post hoc filter criteria and that showed an increase in expression of 5-fold or greater over controls at any time point. NS, change in expression is not statistically significant. Effect of in vitro heat shock refers to the results of our laboratory's previous experiment with normal PBMCs (29). *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria.

Table 9. *Non-HSP genes most strongly downregulated by EHI*

Gene Type	Common Name(s)	GenBank No.	Fold-Change (Subjects/Controls)			Effect of In Vitro Heat Shock
			At presentation	After cooling	At follow-up	
Maximally downregulated at presentation						
Cell growth, proliferation, and differentiation	MAL; mal, T-cell differentiation protein	X76220	0.13	0.29	0.22	Up*
Immune function	FCER1A; Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	X06948	0.039	0.13	0.21	NS
Membrane transport	MS4A1; membrane-spanning 4-domains, subfamily A, member 1; CD20 antigen	X07203	0.090	0.37	0.52 (NS)	NS
Transcription	DRAP1; DR1-associated protein 1 (negative cofactor 2 alpha); Dr1-associated corepressor, mRNA sequence	U41843	0.13	0.39	0.15	NS
	MYC; V-myc myelocytomatosis viral oncogene homolog (avian); c-myc	V00568	0.19	0.50	0.58	NS
Unknown	TBC1D4; TBC1 domain family, member 4; KIAA0603	AB011175	0.073	0.32	0.48 (NS)	NS
Maximally downregulated after cooling						
Cell growth, proliferation, and differentiation	JAG1; jagged 1 (Alagille syndrome)	U77914	0.75 (NS)	0.10	1.9	NS
Immune function	SH2D1A; SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	AL023657	0.71 (NS)	0.053	0.54 (NS)	NS
Metabolism	AGL; amylo-1,6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease type III)	U84011	0.39	0.20	0.34	NS
Neurotransmitter synthesis	GAD1; glutamate decarboxylase 1 (brain, 67 kDa)	M81883	0.40 (NS)	0.088	0.44 (NS)	NS
Signal transduction	ARHH; Ras homolog gene family, member H	Z35227	0.54 (NS)	0.19	0.48	Down*
Transcription	ZNF85; zinc finger protein 85 (HPF4, HTF1)	U35376	0.89 (NS)	0.077	0.61 (NS)	NS
Unknown	KIAA0982	AB023199	0.61	0.16	0.69	Up*
Maximally downregulated at follow-up						
Apoptosis	APG5L; APG5 autophagy 5-like (<i>Saccharomyces cerevisiae</i>); ASP; apoptosis specific protein	Y11588	0.41	0.52	0.15	Up*
Cell growth, proliferation, and differentiation	G0S2; Putative lymphocyte G0/G1 switch gene	M69199	0.033	1.4 (NS)	0.018	Down*
	COIL; Coilin; P80-coilin	U06632	0.69 (NS)	0.26 (NS)	0.11	Down*
	ZRF1; zootin related factor 1; MPP11; MPHOSPH11; M-phase phosphoprotein 11	X98260	0.29 (NS)	0.29 (NS)	0.13	NS
Cytoskeleton	PLAGL1; pleiomorphic adenoma gene-like 1	U81992	1.0 (NS)	0.62	0.19	NS
	AB026190; Kelch motif-containing protein	AB026190	0.45	0.68 (NS)	0.13	NS
	TUBGCP3; tubulin, gamma complex-associated protein 3; GCP3; spindle pole body protein	AF042378	0.33	0.56	0.14	Up
Immune function	PF4V1; platelet factor 4 variant 1; PF4-ALT	M26167	0.50 (NS)	0.91 (NS)	0.031	NS
	TACTILE; T-cell-activated increased late expression	M88282	0.11	0.12	0.041	Down
Membrane traffic and receptor sorting	STX8; Syntaxin 8	AF036715	0.54	0.53	0.11	NS
Membrane transport	KPNB3; karyopherin (importin) beta 3	Y08890	0.34	0.49	0.066	NS
Metabolism	DLAT; dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	Y00978	0.63 (NS)	0.60 (NS)	0.12	NS
	NNT; nicotinamide nucleotide transhydrogenase	U40490	0.47	0.33	0.18	NS
Protein degradation	CTSS; cathepsin S	M90696	1.1 (NS)	0.62	0.18	NS
RNA processing	NUFIP1; nuclear fragile X mental retardation protein interacting protein 1	AL049842	0.13	0.39	0.087	Up*
Signal transduction	PRKCBP1; protein kinase C binding protein 1; RACK7; receptor for activated C-kinase 7	W22296	0.83 (NS)	0.41 (NS)	0.13	NS
	MAP3K12; mitogen-activated protein kinase kinase kinase 12	U07358	0.33	0.42	0.14	NS
Unknown	SMG1; PI-3-kinase related kinase SMG-1	AI610467	0.66 (NS)	0.38 (NS)	0.20	NS
	Hypothetical protein FLJ13110	AL080222	0.59 (NS)	0.49 (NS)	0.032	NS
	HZF12; zinc finger protein 12	AL096747	0.10	0.21 (NS)	0.096	Down*
	KIAA 1718	AC004849	0.32	0.41 (NS)	0.14	NS
	KIAA 0648	AB014548	0.27	0.38 (NS)	0.14	NS

Included are all genes that met our post hoc filter criteria and that showed a decrease in expression of at least 0.2-fold over controls at any time point. NS, change in expression is not statistically significant. Effect of in vitro heat shock refers to the results of our laboratory's previous experiment with normal PBMCS (29). *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria.

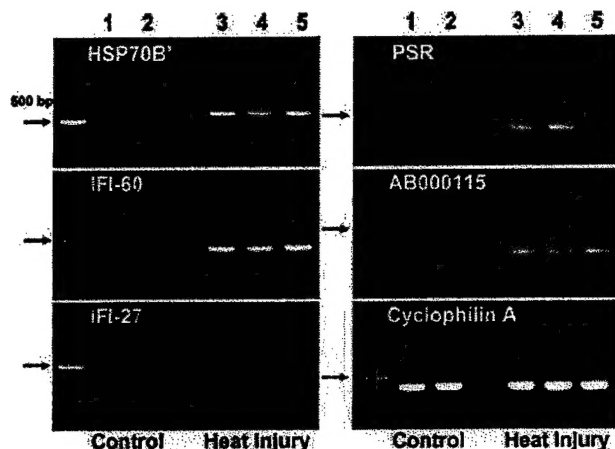


Fig. 2. Confirmatory RT-PCR. The controls are from *subject B*, taken before (lane 1) and after (lane 2) participation in a field-training exercise. The heat-injury samples are from *subject 2*, taken at presentation (lane 3), after cooling (lane 4), and at follow-up (lane 5). Sequences studied were heat shock protein (HSP) 70B'; interferon-induced protein 60 (IFI-60); interferon- α -inducible protein 27 (IFI-27); phosphatidylserine receptor (PSR); chromosome 1 open reading frame 29 (AB000115); and cyclophilin A. The ladder bands occur in 100-bp increments. The arrows point to the bright 500-bp band in each ladder.

firm evidence of an interferon-associated viral process (mononucleosis) on clinical evaluation. The finding of increased expression of interferon-induced genes at the time of presentation may simply be a molecular correlate of antecedent viral infections in our cases but may also identify candidate mediators that might account for the apparent association of EHI with prodromal symptoms suggestive of viral illness. Alternatively, at least some of the increases in expression of these sequences might reflect heat-induced increases in plasma levels of interferons. Further research will be required to establish whether the pathways associated with these sequences participate in the pathophysiology of EHI or whether their increased expression merely serves as markers that identify individuals at increased risk of EHI. Importantly, our microarray evidence to date suggests that the interferon-induced sequences (Table 8) are not directly upregulated by heat itself, because their expression was not significantly affected in our laboratory's previous *in vitro* heat shock experiment, and, indeed, at least three were found to be downregulated *in vitro* (29).

A comparison of the present work to our laboratory's previous *in vitro* work suggests that, as is generally believed, there is a cell type and stressor nonspecific component to the response to stress at the level of gene expression. This nonspecific component includes well-known stress genes such as ubiquitin C, HSP47/colligins 2, and dual-specificity phosphatase-1. The limited microarray evidence our laboratory has produced to date (including the present work) suggests that the number of genes involved in this nonspecific component may be a smaller fraction of the genomic response than previously thought, amounting to no more than ~10–15% of the total number of genes affected by any given environmental stress (27).

Our work confirms and extends, in important respects, the findings made in a mouse model of thermal stress reported recently by Zhang et al. (32). Among other findings, these

authors reported that young mice exposed to two sequential thermal stresses had significant changes in liver gene expression in four principal categories: stress response genes (including HSPs); cell growth, death, and signaling-related genes; antioxidant enzymes and drug metabolism enzymes; and DNA/RNA/protein repair-related genes. Although we found genes related to immune function to be the largest single category affected by EHI (as might be expected from the fact that we studied PBMCs rather than other cell types), we also found a large number of genes to be affected by EHI that are functionally thought to be involved in the cell stress response, signal transduction, metabolism/redox control, and cell growth, proliferation, differentiation, and apoptosis (Tables 8 and 9). This suggests that a number of the functional responses to thermal stress at the level of RNA expression are conserved across species and cell types and adds support to the use of rodent models for the study of heat-related illnesses.

There are several important limitations to this study. First, as an observational rather than an experimental study, the general applicability of its conclusions are limited by the low number of subjects and the presence of significant intersubject variation in severity of illness, prodromal symptoms, clinical course, and timing of sample collection. Accordingly, we are unable to determine the extent to which particular individual sequences might influence severity of disease from the present data. Nonetheless, although the degree of severity was broad, all subjects met clinical criteria for EHI (11). Second, although our controls were matched for gender, ethnic origin, and both season and year of exposure, they were not drawn in parallel with the cases during the events that precipitated EHI. It is, therefore, possible that some of the gene-expression changes identified in our subjects suffering from EHI reflect the effects of variables, such as different types or levels of physical activity, and are not pathophysiologically related to EHI itself. Indeed, increases in leukocyte HSP expression have previously been described in individuals undergoing intense physical activity (6–8, 24), although it can be difficult in these studies to separate the effects of exercise from the effects of exercise-induced increases in body temperature. Third, the low number of replicates (only 2 control chips) coupled with the high number of sequences tested (~12,600) makes it likely that some of the changes in expression we report as significant represent false-positive results. We have attempted to reduce this by application of strict post hoc filter criteria. Fourth, it is possible that some of the changes in gene expression noted reflect a shift in the relative distributions of different subpopulations of circulating PBMCs rather than changes in expression within the same cells. Although this is unlikely to be the case for molecules such as HSPs (which are almost universally expressed by cells in response to thermal stress), it could account for some of the apparent changes in expression of genes known to be highly expressed by specific cell types [e.g., granzyme B, which is selectively expressed by cytolytic T cells and natural killer cells (30)]. Finally, we do not have measurements of plasma interferon levels.

In summary, this is the first large-scale survey of gene expression changes related to EHI or EHS. Our findings suggest that EHI produces gene expression changes that are far more extensive than previously realized. The gene expression signatures that occur during EHI include a classic heat shock response, a small nonspecific cell stress response, and a re-

sponse that cannot be fully explained by our previous in vitro work. This last component involves a significant number of interferon-inducible sequences and might, therefore, account for the known association of EHI with prodromal flu-like symptoms. Finally, PBMCs provide an easily obtainable tissue to study gene expression with EHI and EHS.

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The views, opinions, and findings contained in this publication are those of the authors and should not be construed as an official US Department of the Army or Department of the Navy position, policy, or decision, unless so designated by other documentation.

For protection of test subjects, the investigators adhered to the protections of 45 CFR 46.

Approved for public release; distribution unlimited.

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